IS THERE A COMMON BINDING CENTER IN THE ADP, ATP CARRIER FOR SUBSTRATE AND INHIBITORS?

Amino acid reagents and the mechanism of the ADP, ATP translocator

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1. Introduction

The unique availability of two groups of highly specific inhibitors of the ADP, ATP carrier has made it possible to gain a highly advanced insight into the carrier mechanism on a molecular level. In particular, the binding studies with ADP, ATP [1] and the inhibitors, atractylate (ATR), carboxyatractylate (CAT) [2–4] and bongkrekate (BKA) [3,5–7] led us in elaborate studies since 1969 to the discovery of the reorientation mechanism [6–8] and its molecular version which we called in 1976 the 'gated pore' mechanism [9–11].

As a consequence the carrier has been postulated to exist essentially in two major states, alternating in the translocation process, the 'c-state' where the single binding site is facing the cytosolic side of the membrane and can bind CAT, and the 'm-state' facing the matrix side and can bind BKA. In both states the binding site has a high specificity for ADP and ATP.

Different from our models, a separate binding site of ATR was proposed to be located in a control subunit linked to the carrier subunit [12,13]. Lauquin and Vignais in 1976 maintained that also BKA and ADP bound to separate sites and the carrier forms a ternary BKA–ADP protein complex [14,15]. Our model explicitly excludes this complex, which was first postulated in our binding studies with BKA in 1970 [5], but then explicitly ruled out by an abundance of evidence since 1972 [6,7]. Then, our model of a fixed gated pore in a dimeric protein with binding sites for substrate and inhibitors at the same protein was adopted [16,17]. The localization of the ATR binding site on the outer and that for BKA on the inner membrane face, were also adopted but maintained that both sides are different and do not overlap [16,17]. This modification was based on the finding that UV radiation inhibited binding of ATR but not of BKA to mitochondria [14,16].

Here we stress that also amino acid reagents such as phenylglyoxal [18] may spare binding of BKA when inhibiting that of CAT. However, we show by a comparison with NEM results [9,11,19] that these findings can be accommodated to the single reorienting site model by assuming that these amino acid modifications stabilize the m-state, thus preventing CAT binding. There is no evidence for the localization of these amino acid modifications at the CAT binding site and, consequently, for separate binding sites for CAT and BKA, as postulated in [17].

2. Methods

Phenylglyoxal was obtained from Merck and dissolved in ethanol. [^{3}H]BKA [20] and [^{3}H]CAT [21] were prepared as in [22]. Beef heart mitochondria were prepared as described.

For treatment with phenylglyoxal, beef heart mitochondria were incubated in 40 mM borate buffer at 1 mg protein/ml at 20°C, and for 20 min. The medium also contained 100 μM ATP. Then to each sample, 10 μM [^{3}H]CAT or [^{3}H]BKA were added and after another 5 min mitochondria were centrifuged for 10 min at 8000 × g. Supernatant was carefully removed and the pellets dissolved in SDS for liquid scintillation counting.

3. Results and discussion

The experimental results are a brief excerpt from more extensive studies of the effects of the amino acid reagent phenylglyoxal on the ADP, ATP carrier. Phenylglyoxal has been known to be a relatively specific reagent for arginine [18]. It can be visualized to aim at the binding center of the ADP, ATP carrier which should contain cationic amino acid residues for binding the highly negatively charged ligands ADP, ATP and CAT as well as BKA. No divalent cation is involved in the binding. Three compensating cationic charges have been proposed to be the binding center [9]. In first experiments in fact, phenylglyoxal was found to inhibit the ADP, ATP exchange, although only at high concentrations (unpublished).

In this context some results about the influence of phenylglyoxal on the binding CAT and BKA to mitochondria are presented. These are compared with the influence of the SH-reagent NEM on the binding of both inhibitors, which does not attack the binding center but rather to fix the carrier in the m-state [19].

The influence of phenylglyoxal on the binding of either [^{3}H]CAT or [^{3}H]BKA to beef heart mitochondria is shown in fig.1. Mitochondria were incubated with increasing concentration of phenylglyoxal before addition of CAT or BKA. There is no change of the binding capacity for [^{3}H]BKA whereas the binding of [^{3}H]CAT is increasingly suppressed. In a parallel experiment mitochondria were exposed to increasing amounts of NEM. Similarly the binding of [^{3}H]BKA remains undisturbed whereby the binding of [^{3}H]CAT becomes largely inhibited. In all samples ATP is present to facilitate the transition between the c- and m-states and thus to make the binding centers more accessible to either BKA or CAT by the ATP catalyzed transition between the c- and m-states. The effect of NEM on the differential binding of CAT and BKA has been reported [11]. These results show that phenylglyoxal has principally similar effects on the binding of both ligands as NEM.

We also examined how phenylglyoxal influences the mutual displacement of CAT and BKA (fig.2). Untreated and phenylglyoxal-treated mitochondria were incubated either first with CAT and subsequently with BKA or vice versa. After CAT is added, BKA is still bound to some extent as in these mitochondria not all carrier sites are amenable to CAT. Also some unspecific BKA absorption to the membranes contributes to the binding. Comparison with

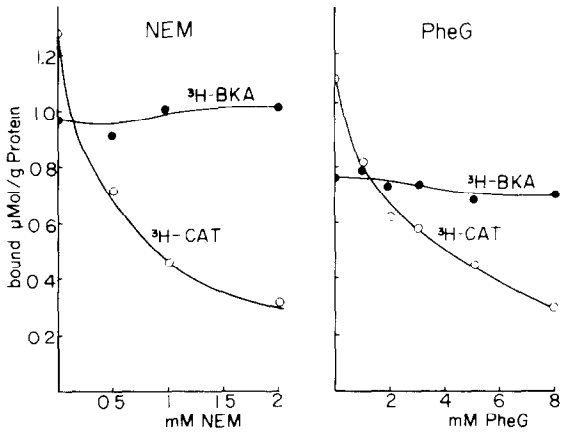

Fig.1. Comparison of the influence phenylglyoxal and of NEM on the binding of CAT and BKA to mitochondria. Beef heart mitochondria were treated with increasing amounts of phenylglyoxal and NEM and after 20 min [^{3}H]CAT or [^{3}H]-BKA under saturating amounts are added. The amount of CAT and BKA bound is measured in the pellets after centrifugation of the mitochondria (PheG, phenylglyoxal).

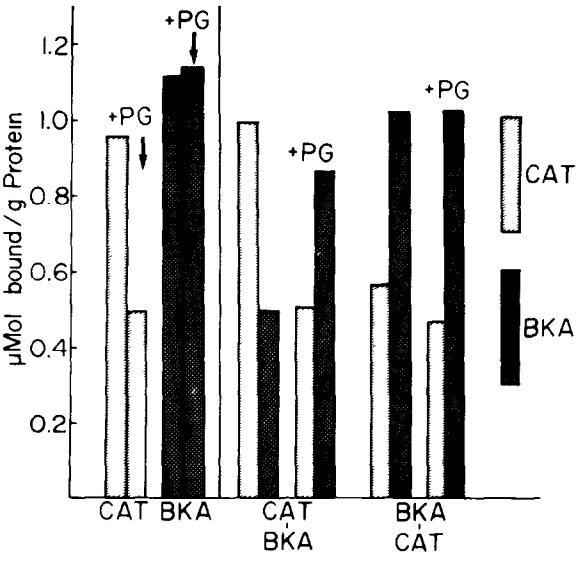

Fig.2. The promotion by phenylglyoxal of BKA binding in favor of CAT in mitochondria. Sequential additions of either CAT and BKA or vice versa on phenylglyoxal-treated or untreated mitochondria. Beef heart mitochondria were pretreated with phenylglyoxal for 20 min. Then the first ligand, and 5 min later the second ligand are added (PG, phenylglyoxal).

the control shows that subsequent addition of BKA does not remove any CAT. However, in the presence of phenylglyoxal the CAT binding is suppressed by ~50% and subsequently added BKA now can bind to a correspondingly larger share of sites. When first BKA is added, CAT binding is diminished and in the phenylglyoxal-treated mitochondria, CAT binding is still more decreased whereas BKA binding remains unchanged. Phenylglyoxal treatment obviously increases the susceptibility to BKA by suppressing CAT binding.

It can be concluded that the arginine reagent phenylglyoxal inactivates CAT but not BKA binding similarly as the SH-reagent NEM and as UV radiation. Among these phenylglyoxal comes closest to a reagent which might attack the binding center since cationic amino acid such as arginine ought to participate in the binding of the anionic phosphate, sulfate or carboxylate groups present in the 3 types of ligands, ADP and ATP, CAT and BKA. This seems to be confirmed by inhibition of CAT binding and of the ADP, ATP exchange (unpublished). At a first sight, the insensitivity of BKA binding to phenylglyoxal then appears to indicate that CAT and BKA, in particular with their anionic groups, occupy different binding centers at the carrier. This argument was advanced in [16] for the results of UV radiation and it now seems to be substantiated more precisely with the arginine reagent.

The pitfalls of this reasoning become apparent by the comparison with the SH-reagent NEM. Essentially the same effect on the binding were found with NEM and with phenylglyoxal or by UV radiation, with the difference that the effect of NEM is more clear-cut. NEM has a further advantage because the specific, ADP-dependent incorporation to SH-groups/carrier molecule can be studied, whereas phenylglyoxal incorporation is rather sluggish and does not permit to differentiate between the 17 arginine groups in the molecule [23]. The rapid reaction of NEM allows to segregate an ADP stimulated from an unspecific reaction.

According to an unreflecting interpretation of these results, also the NEM reactive SH-group should be at the CAT binding site but not at the BKA binding site. A more penetrating interpretation encompasing the various effects of ADP, uncoupler, inhibitors on the NEM interaction with the carrier could be derived from our reorienting site mechanism of the ADP, ATP carrier. This interpretation is shown in

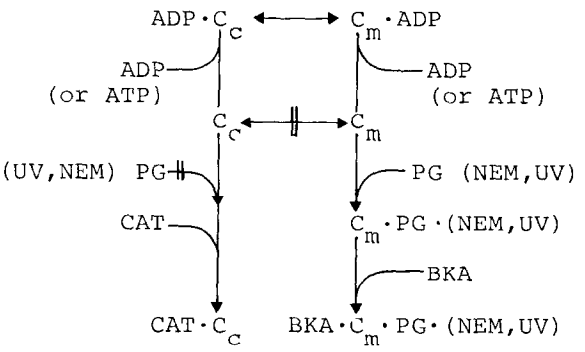

Fig.3. The transition between the c- and m-state of the carrier and interaction of the amino acid reagents phenylglyoxal and NEM. The scheme is a slight modification of that previously published for the reaction of NEM. The reaction of phenylglyoxal with the protein only in the m-state is to represent those arginine groups, the inhibition of which are essentially blocking the transition between the c- and m-state (PG, phenylglyoxal; UV, UV irradiation).

the reaction scheme of fig.3 which represents the reaction step of the reorienting mechanism [7,8] modified to include the interaction of phenylglyoxal. Only a few of the experimental arguments for this scheme can be mentioned here. An extensive account of the NEM-effects will be published shortly (Aquila and Klingenberg). A crucial result is the unmasking of the SH-group to NEM in the presence of ADP. By analogy to the 'unmasking' of BKA binding capability, we postulated that the SH-group becomes reactive only when the carrier is brought into the m-state. All the experiments with the carrier in mitochondria and in the isolated purified state have been in agreement with this concept. The broader evidence is just contrary to what might superficially tell only the binding data, i.e., that NEM reacts with the carrier at the CAT binding site, in the c-state, and that the SH-group is masked in the m-state and therefore NEM does not interact with BKA binding site.

Although with phenylglyoxal the evidence is less powerful, by analogy to the effects of NEM we propose basically the same interpretation. In the m-state one or more arginine groups are unmasked and after reaction with arginine the transition to the c-state and thus binding of CAT but not of BKA is inhibited. The involved arginine groups may not be part of the binding center, similar as the 'essential' SH-group. Actually the relative ineffectiveness of arginine reagents may indicate that these arginine groups are

more peripheral to the binding center. Other supporting evidence for the phenylglyoxal mechanism to be published elsewhere, is also less precise than that for NEM since there are considerably more arginine than SH-groups in the protein (17 to 3) so that the background differentiation of the involved arginine groups is more difficult, moreover, as the arginine groups are much less reactive than SH-groups. Thus an ADP dependence of the phenylglyoxal effect is difficult to demonstrate (unpublished).

We feel that also the UV irradiation falls into the same category, i.e., differential amino acid modification between c- and m-state. In fact here the analogy to NEM is more stringent, as ADP or ATP are found to accelerate the UV inactivation of ATR binding [17]. The authors, however, interpret this unexpected observation by assuming a closer spatial relation of the binding site for ADP to that for ATR than for BKA. In contrast, with the reorientation mechanism the ADP effect is to be predicted and affords a crucial criterion for interpreting the effect as a c–m-state transition.

We can conclude that these results do not contradict, as claimed in [17], but rather support our model of a single binding center for substrate and both inhibitor types. Of course, this does not mean that the binding amino acid regions are identical for all ligands and for both states. For clarification we reproduce the 'gated pore' model of the reorienting carrier sites as it was pictured in 1976 (fig.4A) [11]. A single binding center is visualized to be located in the central channel between the two subunits of the carrier dimer. It is indicated that the center changes strongly its 'configuration' between the two states. This is reflected in the change of specificity for binding CAT to that for binding BKA in the other state. The center has a common core sharing the binding both in the c- and m-state, but additional, different regions along the channels either directed to the c-side or the m-side ought to interact with the substrate. As shown in fig.4B [10] the larger molecules of the inhibitors should occupy additional areas on binding, again retaining a common binding area. At least, the reported differential effects on CAT and BKA binding cannot be interpreted to indicate interaction with the amino acid set exclusively involved in CAT binding, independent of whether there is a common binding center or the binding sites for CAT and BKA are completely separated.

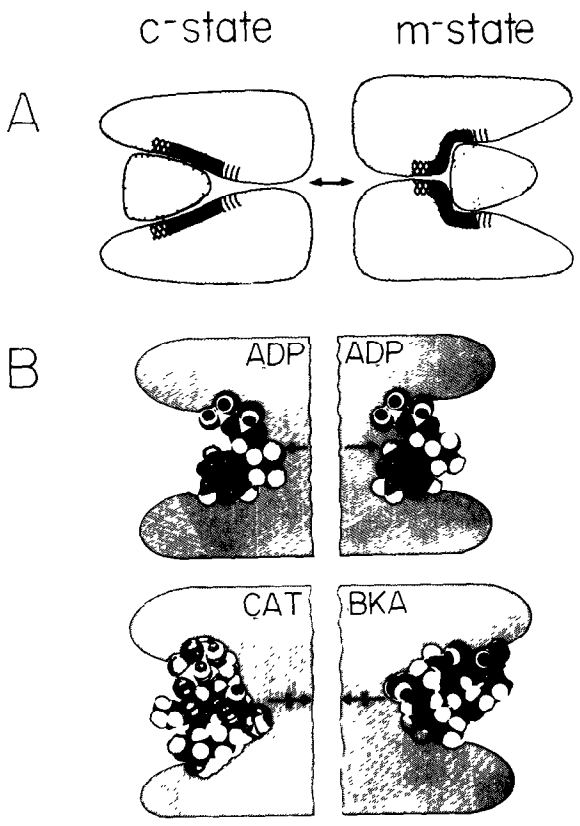

Fig.4. The 'gated pore' model of the dimer ADP, ATP carrier with a common reorienting binding center for substrates and inhibitors as presented in 1975 and 1976. (A) The scheme indicates that in the c- and m-state the binding center employs a common set of amino acids and additional non-common amino acid sets [11]. (B) The binding center of the ADP, ATP carrier in the c- and m-state, with the space-filling molecules of the ligands ADP, CAT and BKA in the c- and m-state [10].

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